HORMONE-SENSITIVE CHOLESTEROL ESTER HYDROLASE OF BOVINE ADRENAL CORTEX

Identification of the enzyme protein

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1. Introduction

Cholesterol ester hydrolase catalyses one of the rate-limiting steps in steroidogenesis in the adrenal cortex, releasing cholesterol from the stores of cholesterol esters in the cytoplasm [1]. The activity of the enzyme is elevated in response to administration of adrenocorticotropic hormone (ACTH) and under conditions of stress, which lead to increased steroidogenesis [2,3]. When partially-purified preparations of the enzyme are incubated in vitro with cyclic AMP, cyclic AMP-dependent protein kinase and ATP-Mg^{2+} the activity of the enzyme increases 3-fold. This activation is blocked by the presence of the protein inhibitor of the cyclic AMP-dependent protein kinase [4]. This evidence strongly suggests that the activity of cholesterol ester hydrolase is regulated by covalent phosphorylation. However, proof of this requires that the enzyme protein be identified and phosphate shown to be incorporated into the protein.

In addition to cholesterol ester hydrolase, adrenal cortex also contains significant levels of a triacylglycerol hydrolase, the activity of which is stimulated by ACTH [5] and there is evidence that both activities are catalysed by the same polypeptide [6]. Evidence to the contrary, such as the selective inactivation of cholesterol ester hydrolase by chlorpyrifos oxone and the differing solubilities of the two enzymes in ammonium sulphate [6] may be at least partly explained by the finding of a second distinct triacylglycerol hydrolase in adrenal cortex [7].

Cholesterol ester hydrolase has been purified 57-fold from bovine adrenal cortex [4]. The major protein component of this preparation has subunit M_r 41 000 and can be phosphorylated by cyclic AMP-dependent protein kinase. However, the specific activity of this preparation is extremely low, being only 0.8 nmol cholesteryl oleate hydrolysed . min^{-1} . mg $protein^{-1}$. This compares with a value of ~30 μmol . min^{-1} . mg^{-1} for the 'hormone-sensitive lipase' purified from rat adipose tissue. This enzyme which hydrolyses triacylglycerol and cholesterol esters has been purified 2000-fold to a state approaching homogeneity, and has a subunit size of 84 000 M_r [8].

Here we report that bovine adrenal cortex cholesterol ester hydrolase activity is catalysed, not by a protein of M_r 41 000 but by a protein of high specific activity and having subunit M_r 84 000. The possible relationship between this enzyme and the hormone-sensitive triacylglycerol hydrolase from adipose tissue is discussed.

2. Materials and methods

2.1. *Materials*

Cholesteryl [1-^{14}C]oleate and glyceryl tri-[1-^{14}C]-oleate were from New England Nuclear, di-[1,3-^{3}H]-isopropylfluorophosphonate ([^{3}H]DFP) was from Amersham International. X-Omat X-ray film was from Eastman Kodak and hydroxylapatite (spheroidal) from British Drug Houses.

2.2. *Determination of enzyme activity*

Cholesterol ester hydrolase and triacylglycerol hydrolase were assayed at pH 7.0 and 30°C, using the ethanol suspension method in [7]. One unit of enzyme is that amount which catalyses the release of 1 μmol oleic acid/min.

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2.3. *Protein determination*

Protein was determined essentially by the method in [9], which involves precipitation of the protein by trichloroacetic acid, prior to determination by the Lowry method [10].

2.4. *Purification of cholesterol ester hydrolase*

The pH 5.2 precipitate fraction was obtained as in [7]. This fraction (20 ml) was applied to Sepharose CL-2B (5 × 75 cm) equilibrated in sodium phosphate (20 mM), EDTA (1 mM), β-mercaptoethanol (15 mM), benzamidine (0.1 mM) (pH 7.0). Cholesterol ester hydrolase was eluted in the excluded volume of the column. Fractions containing enzyme activity were pooled and adjusted to pH 7.4, followed by addition of Triton X-100 to 0.04% (w/v). The enzyme was then applied to DE52 (2.4 × 15 cm), washed thoroughly with sodium phosphate (20 mM), EDTA (1 mM), β-mercaptoethanol (15 mM) Triton X-100 (0.04%, w/v), benzamidine (0.1 mM) (pH 7.4) and then eluted with the same buffer containing NaCl (50 mM). This procedure results in a 60-fold purification of the enzyme.

2.5. *Polyacrylamide gel electrophoresis*

SDS–Polyacrylamide slab gel electrophoresis was done essentially as in [11]. The running gel contained 10% (w/v) acrylamide and the stacking gel 5%. Staining was with Coomassie brilliant blue R. For fluorography, the gels were treated with sodium salicylate then dried prior to exposure to X-ray film [12].

2.6. *Treatment with [^{3}H]DFP*

Fractions containing cholesterol ester hydrolase activity were incubated on ice for 30 min with [^{3}H]-DFP (1.25 vol., 160 μM in propylene glycol), followed by extensive dialysis at 4°C against sodium phosphate (5 mM), β-mercaptoethanol (15 mM) (pH 7.4). Samples were then lyophilised and redissolved in sample buffer for polyacrylamide gel electrophoresis. Occasionally, samples were concentrated immediately after incubation with DFP by precipitation with cold trichloroacetic acid (10%, w/v). The pellet was washed and then dissolved in sample buffer. Both methods gave identical results.

3. Results

Cholesterol ester hydrolase has been purified ~60-fold from the 100 000 × *g* supernatant obtained from bovine adrenal cortex, using a combination of isoelectric precipitation, gel filtration and ion-exchange chromatography in the presence of non-ionic detergent Triton X-100 (section 2.4). The addition of detergent is necessary in order to remove bound phospholipid from the enzyme. As shown in fig.1A, SDS–polyacrylamide gel electrophoresis, followed by staining indicates the presence of several proteins in the preparation, including a major band of 41 000 M_r. This agrees with the report [4] that 57-fold purification of the enzyme yields a major protein of subunit size 41 000 M_r, which was concluded to be the cholesterol ester hydrolase protein [4].

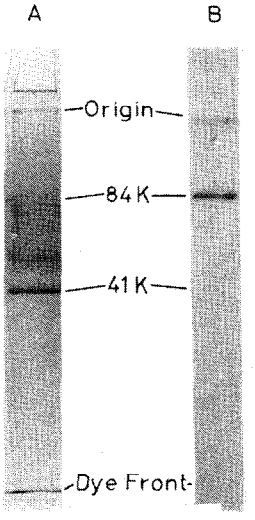

Fig.1. SDS–Polyacrylamide gel electrophoresis of partially-purified cholesterol ester hydrolase. Cholesterol ester hydrolase was purified 60-fold to spec. act. 13 mU/mg and analysed by SDS–polyacrylamide gel electrophoresis: (A) protein stain of sample (10 μg); (B) fluorograph of same sample after pre-treatment with [^{3}H]DFP. Relative molecular mass (M_r) markers employed were phosphorylase *b* (97 000), bovine serum albumin (68 000) and the subunits of bovine heart pyruvate dehydrogenase complex (74 000, 55 000, 41 000 and 36 000).

We have shown that cholesterol ester hydrolase can be inactivated by treatment with phenyl methane sulphonyl fluoride, a reagent which covalently modifies a serine residue at the active site of certain enzymes [7]. We have now investigated the effect on the enzyme activity of di-isopropylfluorophosphonate (DFP), another reagent which selectively modifies active-site serine residues. Treatment with DFP inactivates cholesterol ester hydrolase, and also the tri-

Table 1
Effect of DFP on cholesterol ester hydrolase and triacylglycerol hydrolase activities

Sample	Cholesterol ester hydrolase (mU/ml)	Triacylglycerol hydrolase (mU/ml)
Control	1.5	1.7
DFP-Treated	0.17	0.37

Enzyme was purified as in section 2.4 and then further purified using hydroxylapatite. The overall purification was 200-fold. The enzyme fraction was then treated with DFP (6 μM) as in section 2. The control sample was treated with propylene glycol (1/25 vol.)

acylglycerol hydrolase activity which is present in the preparation (table 1). Following incubation of the enzyme sample with [^{3}H]DFP, SDS–polyacrylamide gel electrophoresis and fluorography indicates that only one protein is covalently modified by that treatment. This polypeptide has M_r 84 000 (fig.1B). No major protein-staining band can be observed at that position on the gel, indicating that the modified protein is a minor component of the protein sample. Furthermore, no radioactivity is detected at the position corresponding to subunit M_r 41 000. These findings strongly suggest that the cholesterol ester hydrolase activity is catalysed not by the 41 000 M_r component but by a 84 000 M_r polypeptide which is a minor component of the 60-fold purified fraction. Furthermore, modification of only one polypeptide results in inactivation of both cholesterol ester hydrolase and triacylglycerol hydrolase, indicating that both activities are catalysed by the same polypeptide. Incorporation of radioactivity into the 84 000 M_r component is blocked by the presence of D,L-α-*O*-benzylglycerol, a water-soluble competitive inhibitor of both cholesterol ester hydrolase and triacylglycerol hydrolase (F.-T. L. and S. J. Y., unpublished). In some preparations a minor band of radioactivity is detected at a position corresponding to subunit M_r 29 000. However, during further purification this polypeptide can be resolved from the enzyme activity (cf. fig.3).

Further evidence that the 84 000 M_r polypeptide is indeed the cholesterol ester hydrolase has been obtained by more extensive purification of the enzyme, using chromatography on hydroxylapatite.

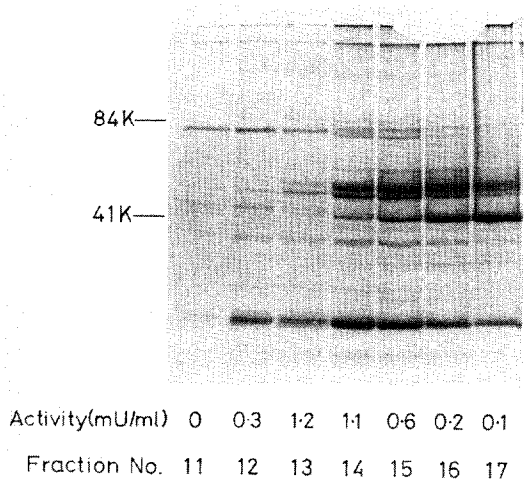

Fig.2. SDS–Polyacrylamide gel electrophoresis of fractions from chromatography on hydroxylapatite: 45 ml 60-fold purified cholesterol ester hydrolase (1.2 mU/ml) in sodium phosphate (5 mM), EDTA (1 mM), Triton X-100 (0.04%, w/v), β-mercaptoethanol (15 mM) (pH 7.4) was applied to a column (1.5 × 12 cm) of hydroxylapatite and washed with 1 column vol. of the same buffer. A linear gradient of sodium phosphate (pH 7.4) (5–200 mM, 200 ml total vol.), containing EDTA (1 mM), Triton X-100 (0.04%), β-mercaptoethanol (15 mM) was applied and 8 ml fractions collected. Aliquots (1 ml) of each fractions were precipitated with trichloroacetic acid (10%) and analysed as in section 2, using staining with Coomassie brilliant blue R.

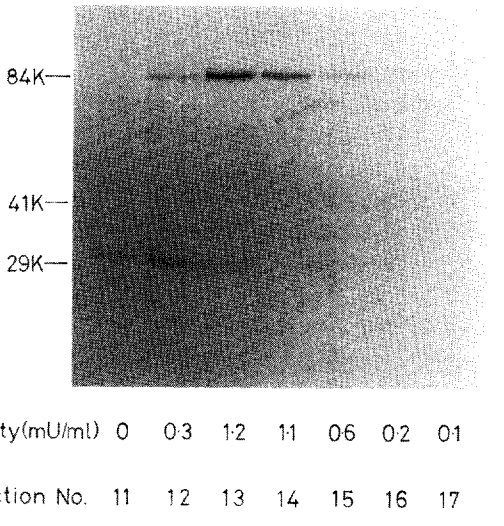

Fig.3. Fluorograph of fractions from chromatography on hydroxylapatite after treatment with [^{3}H]DFP and analysis by SDS–polyacrylamide gel electrophoresis: aliquots (1 ml) of the fractions obtained from hydroxylapatite chromatography of cholesterol ester hydrolase (fig.2) were treated with [^{3}H]DFP, analysed by SDS–polyacrylamide gel electrophoresis and subjected to fluorography as in section 2. Enzyme activity was determined prior to treatment with DFP.

This step succeeds in resolving almost completely the 41 000 M_r component from the enzyme activity (fig.2). In addition, the extent of [^{3}H]DFP incorporation into the 84 000 M_r component in each fraction correlates with the amount of enzyme activity in each fraction (fig.3). A similar result has been obtained from gel filtration chromatography on Sepharose 4B in the presence of Triton X-100 (not shown).

Even in the peak fractions obtained from chromatography on hydroxylapatite, in which the cholesterol ester hydrolase has been purified ~300-fold and has spec. act. 60 mU/mg, the 84 000 M_r polypeptide is only a minor component of the total protein and is scarcely visible after Coomassie blue staining of an SDS–polyacrylamide gel electrophoretogram of the fractions. Assuming that one molecule of DFP is incorporated into each molecule of enzyme it can be estimated, from the incorporation of radioactivity, that after 300-fold purification, the cholesterol ester hydrolase represents only 1–2% of the protein in the purified fraction and that the specific activity of the pure enzyme would be in the region of 5 U/mg. This is 4000-fold higher than that reported in [4], although a part of this discrepancy may be explained by the different assay conditions employed.

4. Discussion

This work presents evidence that cholesterol ester hydrolase activity in bovine adrenal cortex is catalysed by a protein of subunit M_r 84 000. This is in contrast to a report that the enzyme had a subunit size of M_r 41 000 [4]. We too have obtained a partially-purified preparation of cholesterol ester hydrolase which has a major protein component of 41 000 M_r, but adsorption chromatography on hydroxylapatite in the presence of detergent resolves the enzyme activity from that protein. After hydroxylapatite chromatography, the enzyme preparation contains approximately equal cholesterol ester hydrolase and triacylglycerol hydrolase activities. When this preparation is incubated with [^{3}H]DFP, both enzymes are inactivated to a similar extent, and radioactivity is incorporated into only one polypeptide, of M_r 84 000. This suggests that this one polypeptide catalyses both enzyme activities. From the extent of [^{3}H]DFP incorporation it can be estimated that 15 000-fold purification will be necessary to achieve homogeneity and that the specific activity of the purified enzyme against cholesteryl oleate will be ~5 U/mg. The cholesterol ester hydrolase has been purified 100-fold [13] but information concerning the subunit structure of the enzyme, or estimation of the purity of the final preparation were not given [13].

The enzyme which we have partially purified from adrenal cortex bears certain similarities to the hormone-sensitive lipase of rat adipose tissue [8]. The enzyme from adipose tissue also has a subunit size of 84 000 M_r and is inactivated by DFP. It has approximately equal activity against cholesteryl esters and triacylglycerol, the specific activity against cholesteryl oleate being ~30 U/mg. This is in reasonable agreement with the calculated specific activity of the adrenal cortex enzyme, allowing for differences in the conditions and temperature of the assay employed. In addition adrenal cortex cholesterol ester hydrolase is inactivated by similar concentrations of phenylmethane sulphonylfluoride as are required to inactivate hormone-sensitive lipase from pig adipose tissue [7,14].

This raises the interesting possibility that a similar protein may mediate both hormonally-regulated lipolysis in adipose tissue and the supply of cholesterol for steroid synthesis in the adrenal cortex, another process which is under hormonal control. Proof of this will require direct comparison of the molecular properties of the enzymes from adipose tissue and adrenal cortex. Furthermore, identification of the adrenal cortex cholesterol ester hydrolase protein will allow investigation of the control of its activity by phosphorylation and dephosphorylation.

Acknowledgements

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